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Effects of Cold-Treatment on Protein Synthesis and mRNA Levels in Rice Leaves[1]

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ABSTRACT

The effects of cold on protein and RNA metabolism in leaves of rice (*Oryza sativa* L.) seedlings were investigated. Treatment with a diurnal cycle of 15/10°C or 11/6°C for up to 1 week resulted in progressive changes in the protein synthesis pattern after *in vivo* labeling of intact rice leaves with [^{35}S]methionine. These changes were reversed when the seedlings were returned to normal growth temperatures. While *de novo* accumulation of several abundant proteins was suppressed, some polypeptides were consistently found to be cold-induced. Synthesis of ribulose 1,5-bisphosphate carboxylase (Rubisco) was drastically reduced after 7 days of cold. Using immunoprecipitation of Rubisco, evidence was obtained that the suppression was greater for the small subunit (over 90%) than for the large subunit (80%), indicating a partial loss of coordination in their synthesis. Preformed Rubisco as well as other cold-suppressed proteins were stable for up to 7 days at 11/6°C. Cold-sensitive rice cultivars responded with similar but more drastic changes in the protein synthesis pattern when compared to cold-tolerant varieties. The suppression of Rubisco synthesis by cold was shown to result from reduced levels of the mRNAs encoding both subunits; their decrease paralleled the lower protein synthesis of each. The levels of other chloroplast-encoded mRNAs, especially *psaB*, and of the nuclear encoded chlorophyll *a/b* binding protein, also strongly decreased in the cold, whereas the transcripts of the mitochondrial genes *apt9*, *coxIII*, and most nuclear genes analyzed were unaffected or only slightly reduced. These data indicate that some chloroplast functions are disturbed during cold stress. One nuclear gene known to be induced by water stress and ABA (*Rab21*) was also found to be induced by cold treatment.

Many temperate plant species can acquire increased freezing tolerance during exposure to a period of low, nonfreezing temperatures. This process, termed cold-hardening, has been shown to involve changes in gene expression, including the synthesis of new transcripts and polypeptides (6, 13). Cold-induced proteins are proposed to play a role in acclimation of the plants to freezing temperatures. Recently, cDNA clones from cold-induced transcripts have been isolated from alfalfa and shown to be specifically induced by low temperatures (14). On the other hand, many plants of tropical origin do not tolerate cold and can be damaged even at moderate temperatures, *i.e.* below 20°C (4). Membranes have been suspected as a main target of this chilling injury. Loss of membrane fluidity might explain the inactivation of membrane-bound processes such as photosynthesis and ion transport (4). Very little is known, however, about changes in gene expression in cold-susceptible plants.

Cultivation of rice is confined to areas with constantly high temperatures. Nevertheless, remarkable differences exist among rice varieties in their cold susceptibility. We show in this paper that cold treatment of rice seedlings induces major changes in gene expression and that these changes are reversible within 24 h after the return of the plants to control temperatures. The cold-induced changes in protein synthesis and mRNA accumulation demonstrate that suppression as well as induction of gene expression occur in cold-sensitive plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Calmochi-101 is a cold-tolerant japonica variety of rice (*Oryza sativa* L.) developed in California and supplied by the California Cooperative Rice Research Foundation, Biggs, CA. M-202, supplied by the same source, is also cold-tolerant. Lemont, a Texas variety supplied by the USDA, Rice Research, Beaumont, TX, is a less cold-tolerant U.S. japonica variety. Other rice varieties used in this work are Ta Mao Tao (cold-tolerant japonica), Wag Wag, and Peta (cold-sensitive indica), all provided by the International Rice Research Institute in Manila, Philippines. Seeds were imbibed in water overnight and then surface sterilized by immersion in 2.5% NaOCl solution for 30 min; they were rinsed with five changes of sterile water and planted in 200 mL sterilized glass jars containing vermiculite and Hoagland solution. Seedlings were grown in growth chambers with a day/night cycle of 12/12 h, at a light intensity of 80 μE m^{-2} s^{-1}. The control day/night temperature was 32/27°C (50/80% RH). Cold treatments were performed by moving the seedlings into another chamber kept at either 15/10°C (45/80% RH) or 11/6°C (70/90% RH), as indicated in the text. Water stress was applied to the seedlings by omitting the daily water supply to the jars for a period of 3 d, which resulted in the death of the seedlings several hours after the labeling period.

In vivo Labeling and Extraction of Proteins

In vivo labeling of leaf proteins with [^{35}S]methionine (Met) was performed using a nonwounding procedure described in

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(5). Briefly, 10 μCi of [^{35}S]Met (in 10 μL 0.02% Tween 40) were applied to the adaxial side of the second leaf. Three seedlings were labeled per treatment. The labeling time was 8 h during the light period. After the labeling period the leaves were harvested, extensively rinsed with water, frozen in liquid N_2 and stored at −80°C. For SDS-polyacrylamide gels and immunoprecipitation experiments, the proteins were extracted with 100 μL of the extraction buffer described in Oelmüller and Mohr (20) except that it contained 0.2 mM PMSF. For two-dimensional gels, a buffer containing 2% Triton X-100 was used (12). The crude extract was centrifuged at 14,000*g* for 15 min in a microfuge, and the supernatant used either for gel electrophoresis or for immunoprecipitation. Incorporation of [^{35}S]Met into protein was determined by precipitating the cleared extract with 10% trichloroacetic acid. Protein concentration was measured using the Bio-Rad protein assay.

Immunological Methods

For immunoprecipitation of rice Rubisco[3] goat antiserum raised against spinach Rubisco (provided by J. Berry, Stanford) was used. Control experiments with Rubisco purified from rice leaves by centrifugation (3) demonstrated that the holoenzyme is specifically recognized and quantitatively precipitated by the heterologous antiserum (not shown). For quantification of Rubisco synthesis, the enzyme was immunoprecipitated from the cleared extracts as previously described (20), except that no carrier was used. The washed immunoprecipitates were solubilized and loaded onto 15% SDS-polyacrylamide gels. After staining the gels with Coomassie brilliant blue, the bands corresponding to large and small subunit of Rubisco were cut out. The gel pieces were incubated with NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL), and the radioactivity determined by liquid scintillation counting.

RNA Isolation and Blot Hybridization

RNA from rice shoots was isolated as described (10). Poly(A$^+$) RNA selection and RNA blot hybridization was performed using established procedures (11). All hybridizations were carried out under the same, high stringency conditions. The hybridization solution contained 50% formamide, 5 × SSPE (1 × SSPE contains 0.18 M NaCl, 10 mM Na-phosphate [pH 7.7], 1 mM EDTA), 0.1% PVP, 0.1% Ficoll, 0.1% bovine serum albumin, 150 μg/mL denatured salmon sperm DNA, 0.1% sodium dodecylsulfate. The hybridization temperature was 42°C. Restriction fragments were isolated from low gelling agarose and labeled for use as radioactive probes. Specific activities of the probes were between 5×10^8 and 2×10^9 cpm μg^{-1}. For hybridization of the nylon membranes (Nytran, Schleicher and Schuell, Keene, NH) to different probes, probes were removed by 30 min incubation in 6 × SSPE, 50% formamide, at 65°C.

[3] Abbreviations: Rubisco, ribulose 1-5-bisphosphate carboxylase; LSU, large subunit of Rubisco; SSU, small subunit of Rubisco.

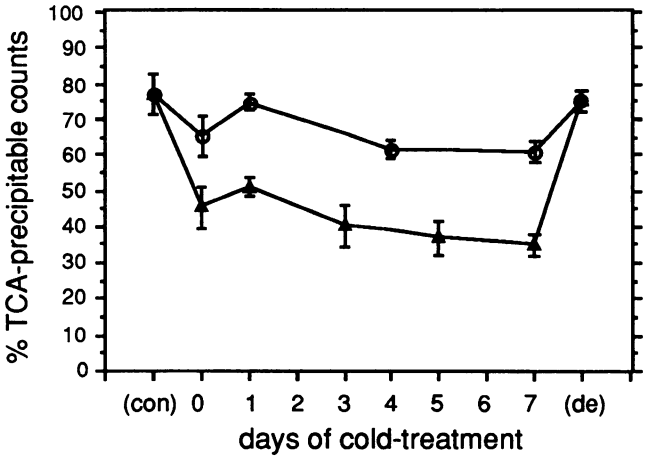

Figure 1. Reduction of [^{35}S]Met incorporation by intact Calmochi-101 rice leaves during cold. Incorporation was determined as a percentage of total radioactivity of cleared leaf extracts. (△), 15/10°C; (○), 11/6°C. Standard errors are shown.

RESULTS

Effect of Cold on [^{35}S]Met Uptake by Intact Rice Leaves

First we sought to determine the temperatures which are required for inducing a severe but nonlethal cold stress to japonica rice seedlings. For Calmochi-101, leaf elongation was strongly reduced at 15/10°C and almost completely inhibited at 11/6°C (data not shown). Both treatments, when applied for up to 14 d, allowed the plants to resume normal growth when returned to control temperatures (32/27°C). Similar results were obtained with M-202; however, Lemont, a more cold-sensitive variety, was severely damaged by the 11/6°C regime.

Incorporation of [^{35}S]Met by the second leaves of Calmochi-101 after different times of cold treatment is shown in Figure 1. Only a moderate decrease was observed at 15/10°C, but after 7 d at 11/6°C, incorporation dropped to less than one-half of the value of control plants. Within 24 h after transfer of the cold-treated plants to control temperatures (deacclimated plants[4]), incorporation was restored to the levels of control plants.

Differential Changes in Protein Synthesis

The proteins of radiolabeled leaf extracts from Calmochi-101 seedlings were separated on one- or two-dimensional polyacrylamide gels and visualized by autoradiography or fluorography. On SDS gels, marked changes in the patterns of protein synthesis were observed during growth at 11/6°C (Fig. 2A). Between 1 and 7 d after beginning of the cold treatment, several polypeptides were apparently suppressed (open arrowheads) whereas others were induced (filled arrowheads). Deacclimated plants, however, displayed a pattern which was almost indistinguishable from that of control plants suggesting that the cold-induced polypeptides are not synthesized at warmer temperatures.

[4] The term 'deacclimation' used in this article does not imply that the cold-induced changes observed in rice leaves contribute to an acclimation, *i.e.* to a better ability of the plants to survive cold. The data presented here do not address this question.

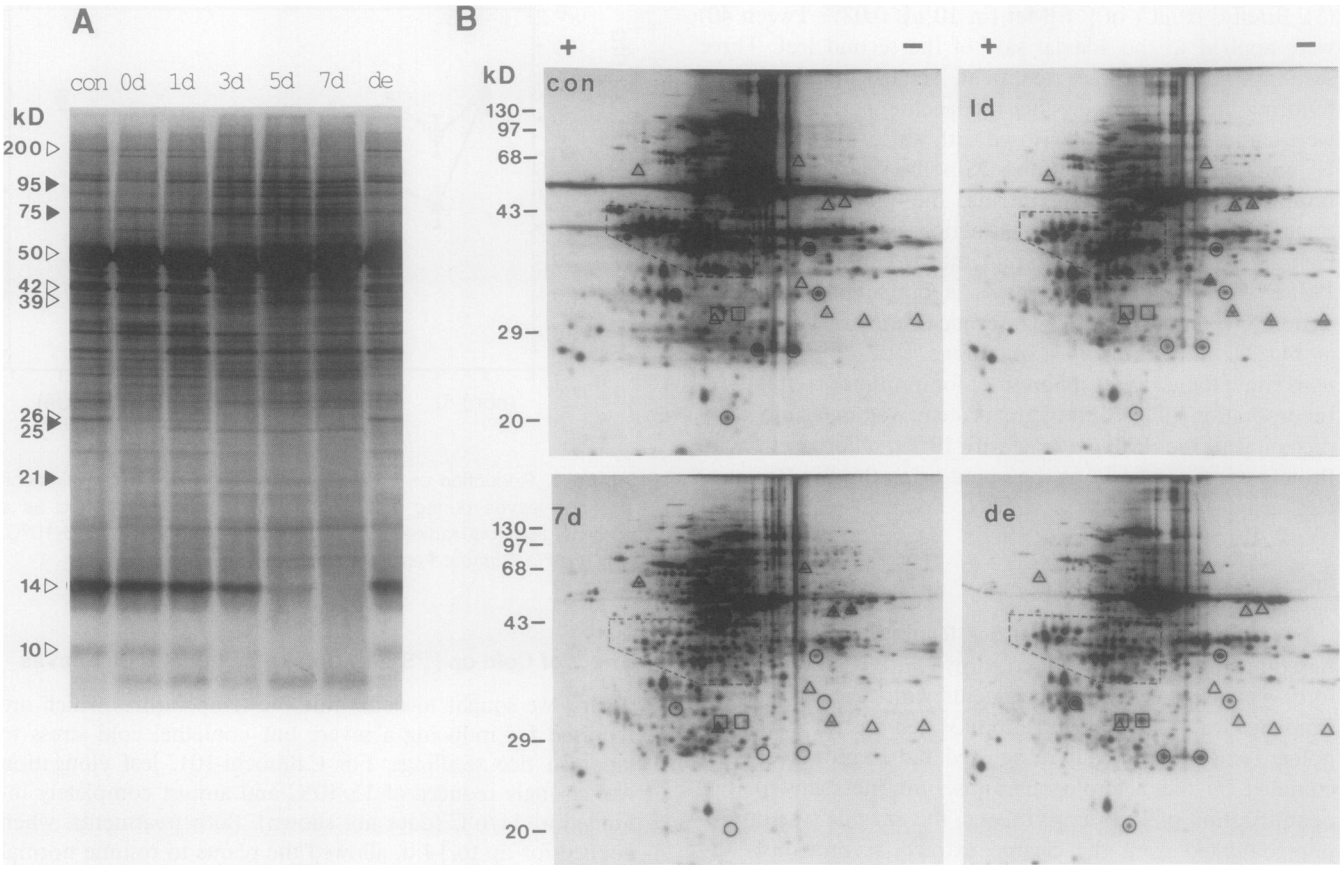

Figure 2. Changes in protein synthesis pattern of Calmochi-101 leaves during cold-treatment (11/6°C). A, Autoradiograph of [^{35}S]Met labeled leaf extracts separated by electrophoresis in a 15% SDS-polyacrylamide gel. Identical amounts of TCA-precipitable radioactivity were loaded in each lane. Samples were from seedlings treated as follows: control (con), labeled immediately after (0 d), or 1, 3, 5, 7 d after the start of cold-treatment, and deacclimated (de). Open arrowheads show cold-suppressed polypeptides and filled arrowheads show cold-induced polypeptides. B, Fluorographs of [^{35}S]Met labeled leaf extracts separated by two-dimensional gel electrophoresis. (○), Cold-suppressed polypeptides; (△), cold-induced polypeptides; (□), deacclimation-specific polypeptides. The dotted box encircles a group of mostly cold-suppressed polypeptides.

Of the proteins suppressed by cold, several were among the most prominent found in the control plants (Fig. 2A). The expression of these proteins (open arrowheads) decreased with duration of cold treatment, until after 7 d, some were barely detectable on the autoradiograph. One polypeptide, with apparent mol wt of 26 kD, was more rapidly suppressed and was found to be reduced in plants labeled immediately after beginning of the cold-treatment. Reproducibly cold-induced proteins (filled arrowheads), with apparent sizes of 95, 75, 25, and 21 kD, increased to maximal levels after 3 to 7 d. Similar results to those shown in Figure 2A were obtained when the plants were grown at 15/10°C, but the changes were less pronounced (not shown). M-202 and Lemont, two other U.S. cultivars, displayed changes in protein synthesis during treatment with 15/10°C which were very similar to those observed with Calmochi-101 (data not shown), suggesting that the response of rice seedlings to cold is similar among japonica varieties. Calmochi-101 showed the best seedling vigor, and was therefore chosen for the additional experiments.

Two-dimensional separation of the leaf extracts largely confirmed and extended the observations obtained with SDS gels (Fig. 2B). Overall, most of the very strongly expressed proteins were progressively suppressed during cold-treatment (circles and dotted box). This made many of the more weakly expressed proteins appear more prominent especially after 7 d at 11/6°C. Nevertheless, several cold-induced protein spots (triangles) were clearly observed. Some of the cold-induced protein spots were stronger after 7 than after 1 d of cold; however, three polypeptides which were consistently seen after 1 d almost completely disappeared after 7 d, indicating that they are transiently induced by cold. As observed with one-dimensional gels, these changes were reversible by 24 h of control temperature as illustrated by the protein pattern of deacclimated plants. Two spots were detected which were unique to deacclimation (Fig. 2B-de, squares). Some of the cold-suppressed proteins could be matched by size between one- and two-dimensional gels. In contrast, cold-induced proteins could not be matched between the two gel systems.

Cold-Induced Changes in Protein Synthesis are Specific

To see if the observed protein synthesis pattern is specific for cold-treated seedlings, we compared it to the labeled protein pattern of seedlings subjected to water stress, abscisic acid-treatment, or NaCl-treatment (Fig. 3). None of these treatments resulted in protein patterns similar to those obtained after cold treatment.

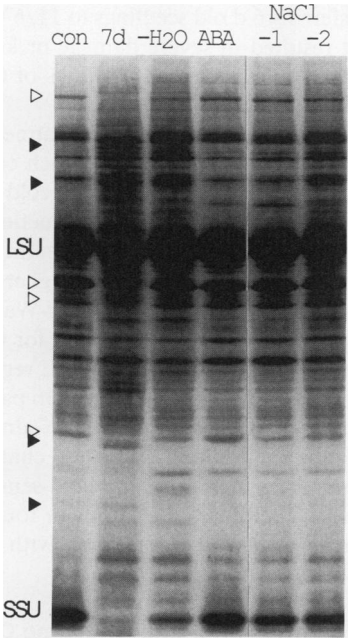

Figure 3. Specificity of cold response. Calmochi-101 seedlings were treated as follows before labeling: controls (con), 7 d at 11/6°C (7d), water stress for 3 d ($-H_2O$), 10 μM ABA in the growth medium for 24 h (ABA), 200 mM NaCl for 1 d (NaCl-1), 200 mM NaCl for 2 d (NaCl-2). Polypeptides induced or suppressed by cold are marked as in Figure 2A.

Cold-Suppression of Rubisco Synthesis

Two of the predominantly synthesized polypeptides in control leaves, with apparent mol wt of 50 and 14 kD, were strongly repressed after 1 week of cold-treatment (Fig. 2A). From their size as well as from their abundance on Coomassie blue-stained gels, we hypothesized that they were the LSU and SSU of Rubisco. This enzyme is by far the most abundant, soluble leaf protein and plays a key role in photosynthetic carbon assimilation. We therefore decided to study the effects of cold on the synthesis of this enzyme in detail. In order to quantitatively determine the decrease in Rubisco synthesis, the holoenzyme was purified by immunoprecipitation. After SDS gel electrophoresis of the precipitate to separate LSU and SSU, the bands for each subunit were excised and individually counted (Fig. 4, A and B). While synthesis of both subunits gradually decreased, SSU was more strongly reduced (>90%) than LSU (80%) after 7 d at 11/6°C.

To further analyze the different effect on SSU and LSU synthesis of cold, we calculated the ratio of [^{35}S]Met incorporation per Met residue. From the available sequences for rice LSU (15) and SSU (25) genes, the number of Met residues could be determined: 11 for LSU and 2 for the processed form of SSU. Because the subunit composition of Rubisco is equimolar (L_8S_8), the expected ratio of [^{35}S]Met incorporation (LSU:SSU) is 5.5:1. The observed ratios of control and deacclimated leaves are in agreement with this value, but the ratios are 2 to 3 times higher after 5 and 7 d of cold-treatment (Fig. 4B, bottom). These data indicate that the coordination of LSU and SSU synthesis might be partially lost under cold conditions.

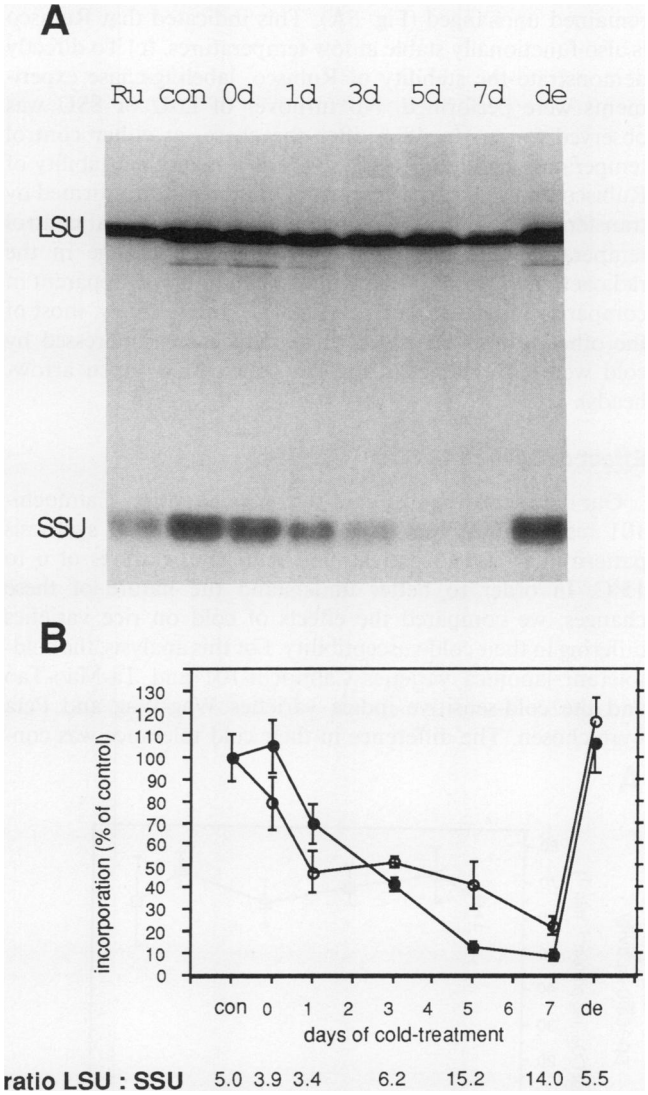

Figure 4. Suppression of Rubisco synthesis by cold-treatment. A, Fluorograph showing the Rubisco subunits after immunoprecipitation and separation in a 15% SDS-polyacrylamide gel. Immunoprecipitates from leaf extracts containing the same amounts of TCA-precipitable radioactivity were loaded in each lane. Rubisco purified from [^{35}S]Met labeled leaves was loaded as a control (Ru). B, Noncoordinated decrease of LSU- and SSU-synthesis during cold stress. Standard errors are shown. The bottom lane shows the ratios of [^{35}S]Met radioactivities incorporated into both subunits.

Stability of Rubisco during Cold Treatment

For the experiments described above, the labeling period was 8 h. One possible explanation for the decrease in the synthesis of Rubisco and other proteins during cold would be an increased protein degradation. The following experiments were performed to address this question: (a) Using quantitative immunodiffusion of leaf extracts in agarose containing anti-Rubisco serum, the concentration of Rubisco (per μg leaf protein) was found to be unchanged for up to 10 d at 11/6°C (data not shown). A major increase in Rubisco degradation was therefore unlikely. (b) Enzyme assays were performed with leaf extracts from control and from cold-treated plants. During a 1 week period, the specific activity of Rubisco

remained unchanged (Fig. 5A). This indicated that Rubisco is also functionally stable at low temperatures. (c) To directly demonstrate the stability of Rubisco, labeling-chase experiments were performed. No turnover of LSU or SSU was observed for up to 48 h after the chase, at either control temperature or 11/6°C (Fig. 5B). The remarkable stability of Rubisco under the experimental conditions was confirmed by transferring seedlings which had been radiolabeled at control temperatures into the cold for 7 d. Little change in the radioactivity present in the Rubisco subunits was apparent in comparison to control plants (Fig. 5C). Interestingly, most of the other prominent polypeptides which are suppressed by cold were also stable during the cold period (open arrowheads).

Effect of Cold on Different Varieties

Our data show that the cold-tolerant rice variety Calmochi-101 responds with several changes in its protein synthesis pattern after extended treatment with temperatures of 6 to 15°C. In order to better understand the nature of these changes, we compared the effects of cold on rice varieties differing in their cold-susceptibility. For this analysis, the cold-tolerant japonica varieties Calmochi-101 and Ta-Mao-Tao and the cold-sensitive indica varieties Wag-Wag and Peta were chosen. The difference in their cold tolerance was confirmed by transferring 6 d old seedlings to 11/6°C for 2 weeks. This treatment resulted in severe damage or killing of Wag-Wag and Peta seedlings, whereas seedlings of Calmochi-101 and Ta-Mao-Tao were little affected.

Incubation at 15/10°C, a milder cold treatment, resulted in suppression of both Rubisco subunits which correlated with the general susceptibility of the cultivars to cold (Fig. 6). Wag-Wag and Peta showed a similar, drastic reduction of LSU and SSU synthesis after 7 d while the cold-tolerant varieties Calmochi-101 and Ta-Mao-Tao maintained higher levels of Rubisco synthesis. In fact, the values for Wag-Wag and Peta at 15/10°C were similar to those determined for Calmochi-101 after treatment with the more severe 11/6°C regime (Fig. 2A). Examination of the one-dimensional protein pattern revealed that most of the changes described for Calmochi-101 are observed with all varieties, and that these changes are more pronounced and occur earlier in the cold-sensitive varieties (data not shown). Therefore, alterations in total protein and Rubisco synthesis seem to be correlated with the degree of cold stress experienced by the plant.

Rubisco mRNA Levels are Reduced during Cold-Treatment

The results thus far establish that cold stress leads to a strong suppression of Rubisco protein synthesis in rice leaves.

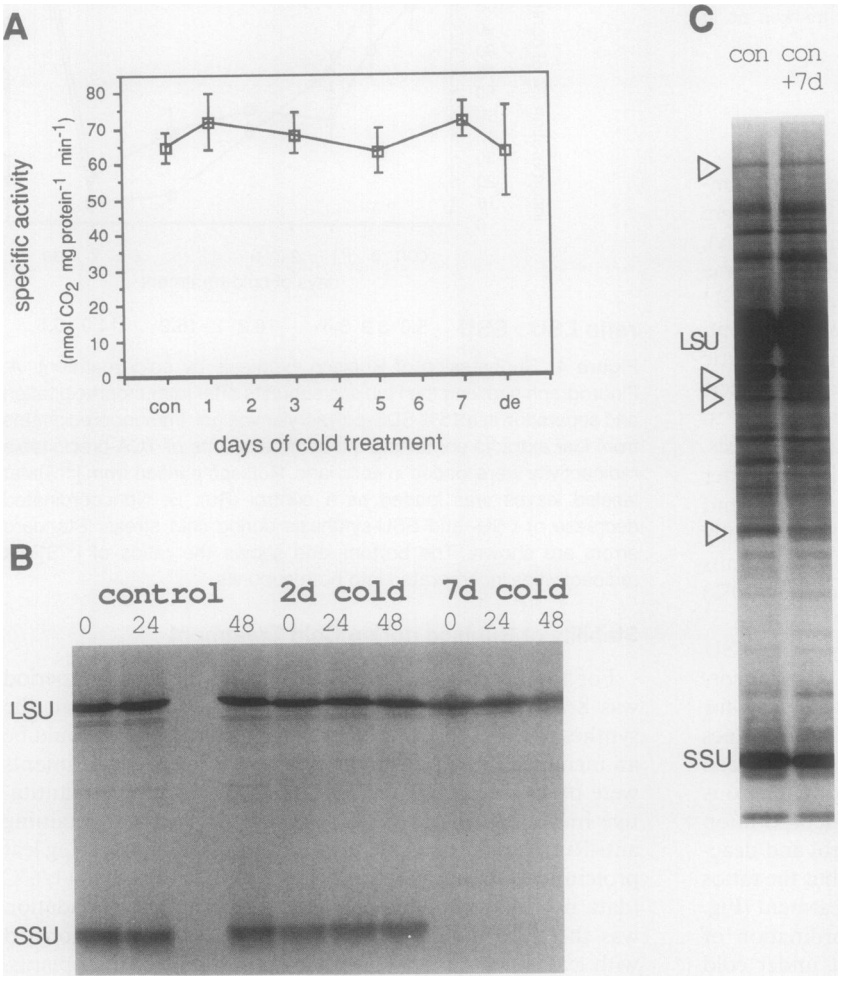

Figure 5. Stability of Rubisco during cold stress (11/6°C). A, Stability of Rubisco activity in the cold. Each value for CO_2 fixation is the mean of three or four independent measurements performed in duplicates. Standard deviations are shown. B, Gel fluorographs with Rubisco-immunoprecipitates from leaves after labeling-chase experiments. The labeling time was 8 h for seedlings grown at control temperature (con), and 24 h for seedlings shifted to cold 2 d or 7 d prior to the chase treatment. The chase was performed, after rinsing the radiolabeled parts of the intact leaves with several changes of H_2O by applying 15 μL of a 1 mM Met solution (containing approximately 1000-fold molar excess of Met compared to the [^{35}S]Met labeling solution) to the previously radiolabeled part of the leaves. Seedlings were harvested immediately after (0), 24 h (24) or 48 h (48) after the chase. Similar aliquots of extracts from the different treatments were used for immunoprecipitation. C, Stability of radiolabeled leaf proteins during 7 d of cold treatment. Seedlings labeled at control temperature for 8 h were immediately harvested (con) or cold-treated for 7 d (con + 7 d). Cold-suppressed polypeptides are marked as in Figure 2A.

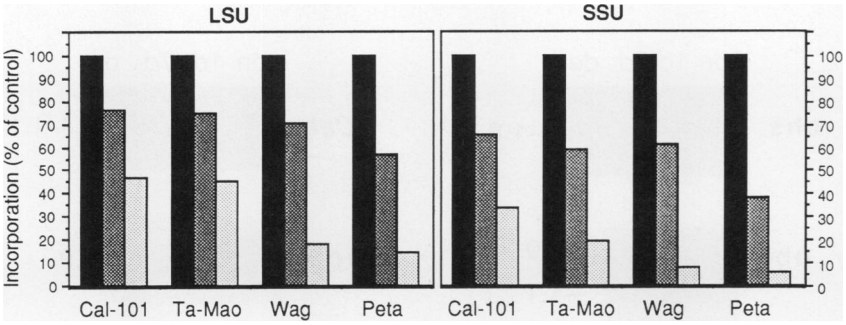

Figure 6. Decrease in Rubisco expression in different rice varieties at 15/10°C. Black bars: controls; dark shaded bars: after 1 d cold; light shaded bars; after 7 d cold.

To learn more about the mechanism of this suppression, the mRNA levels of the genes encoding LSU (*rbcL*) and SSU (*RbcS*) were analyzed. As shown in Figure 7, the abundance of the transcripts for both subunits progressively decreased during cold treatment, but resumed control levels after 24 h of deacclimation. The changes in RNA amount were similar to those observed with the synthesis of both subunits. For example, the transcripts of *RbcS* decreased to lower relative levels than those of *rbcL* as was found in comparing SSU to LSU (Fig. 4). The reduction in *RbcS* mRNA was not simply the result of a general decrease in total cytoplasmic mRNA during cold, because a similar result was obtained by using poly(A^+) RNA for Northern hybridization (Fig. 8A). Thus, the SSU mRNA decreased as a percentage of total cytoplasmic mRNA. These data show that the cold-suppression of Rubisco synthesis is mainly the result of reduced accumulation of Rubisco mRNAs.

Behavior of Other mRNAs in the Cold

The profound effects of cold on expression of Rubisco and other, unidentified proteins prompted us to search for other cold-induced changes at the level of RNA accumulation. By using probes from genes encoded by each of the three genetic compartments of the plant cell (nucleus, plastids, mitochondria), a more general understanding of the effects of cold treatment might be obtained. The probes used in this study are described in Table I.

Among the nuclear-encoded genes tested, three different types of responses to cold treatment were observed (Fig. 8A). Most of the transcripts, such as those encoding β-tubulin, chalcone synthase, ubiquitin, and histone 3 (not shown) remained unchanged or were only slightly reduced. In contrast, the transcripts encoding the Chl *a/b* binding protein of the light harvesting complex (*Cab*), the already mentioned SSU and sucrose synthase were strongly suppressed. The *Cab* transcripts were distinguished by the fact that they dropped to approximately 10-fold lower levels within 24 h after the beginning of cold-treatment. Finally, an increase in the transcript levels of *Rab21*-like gene during cold was found; *Rab21* has previously been characterized to be desiccation- and abscisic acid-induced (17; P Chandler, personal communication). With the probe used, which was isolated from barley, at least three different transcripts were detected; two of them were induced by cold. The smallest of these transcripts corresponds in size to the *Rab21* transcript described (17).

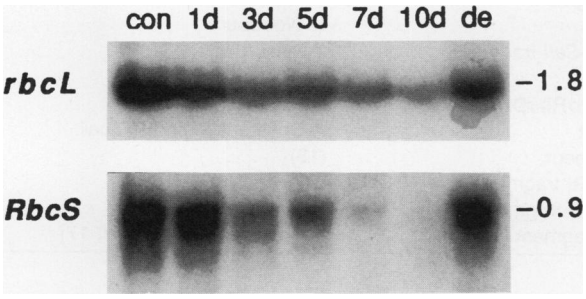

Figure 7. Cold-induced decrease of Rubisco mRNAs. Twenty μg of total leaf RNA was loaded per lane. The same filter was first hybridized to *rbcL* and, after washing off the probe, hybridized to *RbcS*. Seedlings were deacclimated after 10 d at 11/6°C.

The data for the nuclear-encoded transcripts showed that the genes encoding chloroplast proteins (*RbcS* and *Cab*) are specifically and strongly suppressed whereas transcript levels of most other genes are little affected by cold. Both *RbcS* and *Cab* are known to require functional plastids for their normal expression (19). It thus appeared likely that some chloroplast functions were impaired by the cold-treatment. To test this idea, the expression of additional chloroplast-encoded genes was monitored (Fig. 8B). Similar to *rbcL* and *RbcS*, *psaB* transcripts were found to be strongly reduced after 7 d whereas a moderate decrease was observed for *atpE* and *psbB*. After deacclimation, the mRNA levels did not completely recover to the levels found in control seedlings. On the other hand, *psbA* mRNA decreased only slightly. No significant changes were observed with the mitochondrial messages *coxIII* and *atp9* during cold treatment (Fig. 8C).

DISCUSSION

Our work has demonstrated that leaves of rice seedlings respond to cold stress with distinct changes in gene expression. These are superimposed on the general reduction of metabolism that is a consequence of lowered temperature. The effects of cold on protein synthesis did not become apparent until after 24 h and became more pronounced with time (Fig. 2A). This is unlike the heat shock response which occurs very quickly and in a transient manner, involving immediate repression of most nonheat shock proteins (8). Studies with spinach have also shown that the effects of heat and those of cold are quite distinct from each other (6).

One- and two-dimensional gel electrophoresis of protein extracts was used to survey cold-induced changes; we demonstrated that a number of proteins were strongly suppressed by cold. Many of these were among the most strongly ex-

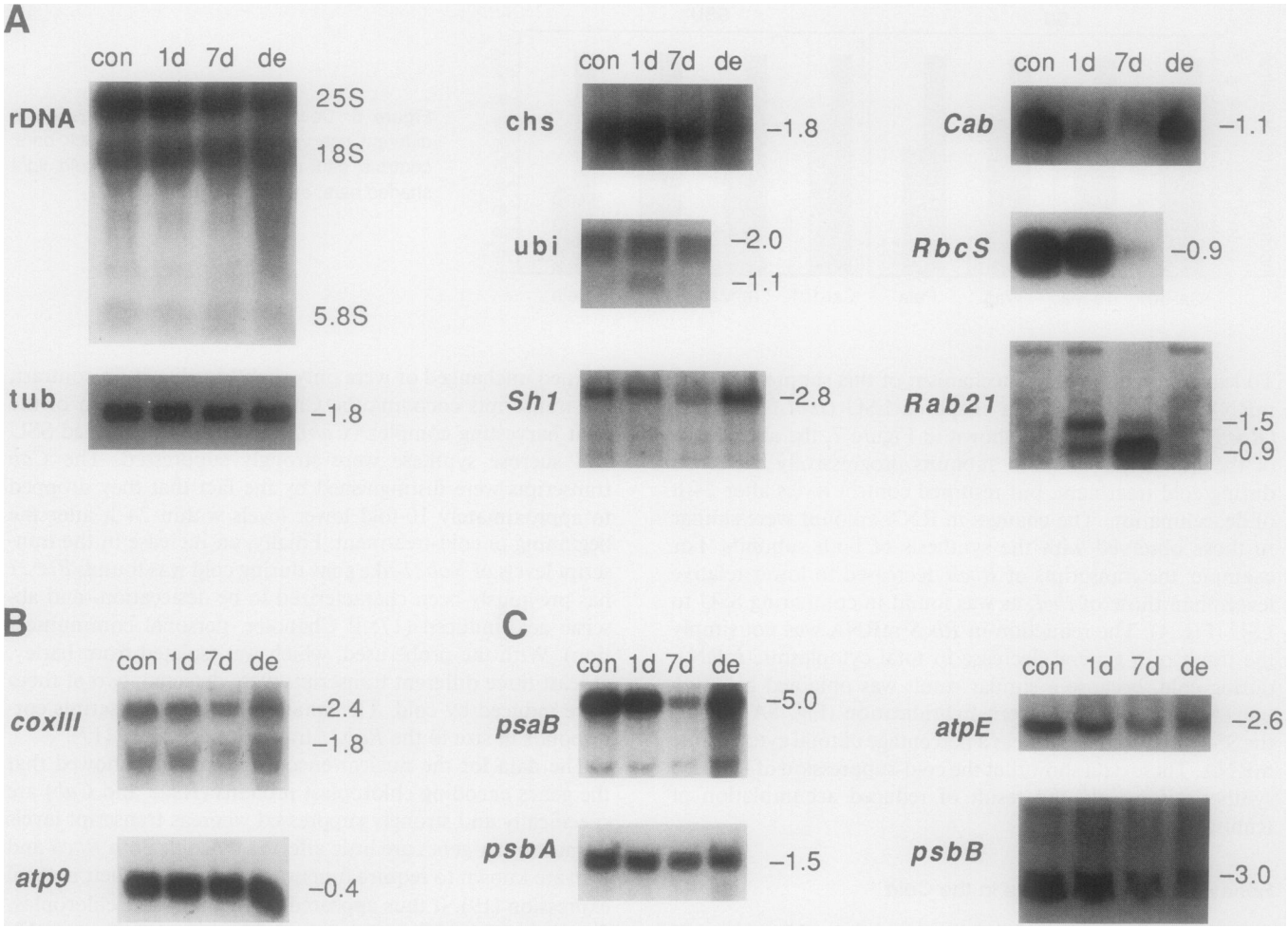

Figure 8. Effect of cold (11/6°C) on levels of mRNAs encoded by the nucleus (A), plastid (B), and mitochondria (C). The filters hybridized to most nuclear clones contained 1 μg of poly(A⁺) RNA per lane, whereas the filters hybridized to rDNA, chalcone synthase, to plastid and to mitochondrial clones contained 20 μg of total shoot RNA per lane. Mol wt (in kb) are shown for the major hybridizing bands.

Table I. *Cloned Fragments Used in Hybridization Analysis*

Gene	Description	Source/Reference
atp9	Maize, 0.5 kb *Bam*HI-*Hin*cII fragment	(16)
coxIII	Maize, 1.39 kb *Bgl*II-*Xba*I fragment	RM Mulligan
rbcL	Rice, 564 bp *Pst*I fragment	(15)
psbA	Pea, 532 bp *Eco*RI-*Pst*I fragment	N Woodbury
psaB	Spinach, 1.6 kb *Bam*HI fragment	N Woodbury
atpE	Spinach, 420 bp *Eco*RI – *Xba*I fragment	N Woodbury
psbB	Spinach, 338 bp *Bam*HI fragment	N Woodbury
RbcS	Maize genomic DNA, 368 bp *Nco*I-*Sal*I fragment	T Nelson
Cab	Maize cDNA, 0.9 kb *Pst*I fragment	W Taylor
Ribosomal DNA	Soybean genomic DNA, 9.2 kb (*Eco*RI) rDNA repeat	(22)
Ubiquitin	Maize cDNA, 975 bp *Pst*I fragment	A Christensen and P Quail
Chalcone synthase	Maize cDNA, 1461 bp *Eco*RI fragment	(18)
Sucrose synthase (*Sh1*)	Maize genomic DNA, 10.5 kb *Eco*RI fragment	LC Hannah
β-Tubulin	*Chlamydomonas* cDNA, 264 bp *Pst*I fragment	(26)
Rab21	Barley cDNA, about 200 bp *Pst*I fragment	P Chandler (see also ref. 17)

pressed leaf proteins in control plants. Cold treatments were not lethal, because in deacclimated seedlings, growth resumed and protein synthesis returned to a pattern very similar to that of the control seedlings. Only two proteins specific for deacclimated leaves were detected on two-dimensional gels (Fig. 2B, de). We did not analyze if these proteins are truly deacclimation-specific or rather related to the higher physiological leaf age of deacclimated plants. On one-dimensional

gels, at least four polypeptides (of 95, 75, 25, and 21 kD) were found to be cold-induced. They were not synthesized in deacclimated plants, showing that their synthesis was not the result of developmental changes during cold-treatment. On two-dimensional gels, several cold-induced polypeptides were also found (Fig. 2B) which were of different molecular mass than those found on SDS-gels. This might be explained by the different extraction buffers and the different mechanisms of protein solubilization and separation employed in these gel systems.

The effects on protein synthesis of 15/10°C were qualitatively similar to those described for 11/6°C; however, the extent of the response was less at higher temperatures. Furthermore, very similar patterns of protein synthesis after cold-treatment were observed for five different rice varieties (data not shown). Therefore, rice seedlings appear to respond to low temperatures with a common and gradual change in their protein metabolism.

Both subunits of Rubisco were found to be strongly suppressed during extended cold-treatment. This led to an overall shift of [^{35}S]Met incorporation into other leaf proteins. Whereas in control plants, up to 50% of the label was incorporated into Rubisco, this proportion sank to less than 10% after 1 week at 11/6°C. Because the same amount of TCA-precipitable radioactivity from each treatment was loaded on the gels or used for immunoprecipitation, the values for suppression of Rubisco synthesis are an underestimation. This is because Rubisco, as well as other cold-suppressed proteins, constitute a major proportion (more than 50%) of the total incorporated radioactivity in control plants but a sharply reduced proportion in cold-treated plants. Therefore, with respect to Rubisco, extracts from cold-treated plants were 'overloaded.'

Rubisco synthesis appears to be very sensitive to many environmental factors. It has been shown recently, using several techniques, that different stress factors can lead to a suppression of Rubisco or SSU synthesis; for example, infection by pathogens (7), heat shock (24), and cold treatment (13). The results presented here quantitatively describe the suppression of LSU and SSU synthesis in rice seedlings exposed to low temperatures. Our data indicate that coordination of their synthesis is partially lost during cold stress. This conclusion, however, needs to be supported by more data, such as the demonstration of an accumulation of free labeled LSU during cold. Despite its reduced synthesis, Rubisco was found to be almost completely stable and functionally active during up to 1 week in the cold. This is in contrast to the situation which occurs in senescing leaves, where the large decrease in Rubisco synthesis is accompanied by increased turnover (21). Cold, therefore, does not lead to premature senescence, but rather to a reversible arrest of synthesis of this major cell component. Our interpretation is supported by the observation that other prominent, cold-suppressed proteins are also stable in the cold and at control temperatures (Fig. 5C; M Hahn, unpublished results). In this sense, cold treatment simply puts some aspects of cell differentiation 'on hold,' without accelerating protein degradation; this is very different from heat shock in which protein turnover is enhanced. Other evidence in agreement with this interpretation is the lack of induction by cold of ubiquitin mRNA (Fig. 8), which can be induced by heat shock (1; A Christensen, P Quail, personal communication).

When different rice varieties were subjected to the same cold treatment, cold-susceptible seedlings showed a stronger suppression of Rubisco and other proteins than did cold-tolerant varieties. Interestingly, the 25 kD and especially the 21 kD protein were much more strongly induced at 15/10°C in Wag-Wag and Peta than in Calmochi-101 and Ta Mao Tao (not shown). Therefore, the changes in protein synthesis pattern reflect a stress response which is displayed earlier or at higher temperatures in cold-susceptible genotypes. Nevertheless, protein synthesis appeared to be changed by cold-treatment in a unique manner. Seedlings subjected to severe desiccation, abscisic acid treatment or NaCl-induced salt stress, exhibited protein synthesis patterns which were largely different from those obtained with cold-treated seedlings (Fig. 3).

The transcript levels of the Rubisco genes were found to be reduced with kinetics similar to the synthesis of their respective (mature) translation products (Figs. 4A and 7). Consequently, cold acts at the level of transcription and/or transcript stability to effect the suppression of Rubisco synthesis. Interestingly, the genes for the Rubisco subunits are encoded in two different cellular compartments, the chloroplast (*rbcL*) and the nucleus (*RbcS*). Nevertheless, it appears that their expression is still linked, at least to some extent, during cold-treatment.

In addition to Rubisco mRNAs, a number of other transcripts from chloroplast-encoded, mitochondrial, and nuclear genes were screened for cold-induced changes in their abundance (Fig. 8). This approach can help pinpoint the primary targets of cold stress in rice. In general, the results shown in Figures 7 and 8, demonstrate that cold stress induces differential changes in gene expression in cold-sensitive plants on the level of mRNA accumulation, as was shown for protein synthesis.

The majority of transcripts analyzed were found to be either unaffected or slightly suppressed by 1 or 7 d of cold. In this group were the transcripts for chalcone synthase, which can be induced by wounding/infection, respectively (9). A more strongly cold-suppressed transcript, encoding sucrose synthase, has previously been shown to be induced during anoxia (23). Therefore, cold does not seem to induce genes which are activated by these other stresses. Nevertheless, cold-induced accumulation of *Rab21* showed that at least some of the genes induced by desiccation and abscisic acid respond in a similar manner to low temperatures. Whether this is the result of cold *per se* or of chilling-induced water stress remains unclear. We observed that, despite the high RH at 11/6°C, the first evidence for cold-induced damage to rice seedlings was a drying of the leaf tips; tissue damage increased with the length of cold-treatment. On the other hand, *Rab21* induction occurred within 24 h after beginning the cold-treatment, when the seedlings looked perfectly normal.

The most strongly cold-suppressed mRNAs were either encoded by chloroplast genes (*psaB*, *psbB*, *rbcL*, *atpE*) or by nuclear genes coding for chloroplast proteins (*RbcS*, *Cab*). Only one chloroplast-encoded transcript, *psbA*, was little changed. These data indicate that chloroplast functions are specifically affected during cold-treatment. The drastic

suppression of *psaB* (PSI) transcripts, as opposed to those of *psbA* and *psbB* (PSII) indicates that the two photosystems might respond differently to cold stress. Synthesis of PSI may be a particularly cold-sensitive component within the chloroplast or, conversely, high turnover of PSII may require continual protein synthesis. Experiments are in progress to define photosystem turnover to address these possibilities. The nuclear genes *RbcS* and *Cab* are both known to require, besides light, functional plastids for their expression (see ref. 19 for review). The plastidic factor assumed to mediate activation of these and other nuclear genes (19), appears to be depleted in cold-treated rice leaves. The depletion seems to occur quite rapidly, since *Cab* transcript levels are strongly reduced within 24 h of cold treatment. The difference from the more slowly decreasing *RbcS* transcripts might be explained either by a different requirement of the plastidic factor or by different stability of these mRNAs. Interestingly, a polypeptide(s) of approximately 26 kD was found both in one- and two-dimensional gels which corresponded in size to the *Cab*-encoded Chl *a/b*-binding protein and which was rapidly suppressed during cold treatment (Fig. 2, A and B). In a previous report, it has been shown that cold-treatment of tomato leaves leads to a suppression of the *Cab*-encoded protein (2).

The reduction in *Cab* transcript levels during cold precedes or, at least, coincides with the changes in the levels of chloroplast-encoded mRNAs. It therefore appears that the chloroplasts are still relatively normal in their competence for gene expression while they have lost the ability to produce the factor necessary for expression of nuclear genes such as *Cab* and *RbcS*. Thus, it will be of considerable interest to analyze more precisely the effects of cold on chloroplast functions.

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LITERATURE CITED

1. **Burke TJ, Callis J, Vierstra R** (1988) Characterization of polyubiquitin genes in *Arabidopsis*. Mol Gen Genet **213:** 435–443
2. **Cooper P, Ort DR** (1988) Changes in protein synthesis induced in tomato by chilling. Plant Physiol **88:** 454–461
3. **Goldwaithe JJ, Bogorad L** (1971) A one-step method for the isolation and determination of leaf ribulose-1,5-diphosphate carboxylase. Anal Biochem **41:** 57–66
4. **Graham D, Patterson BD** (1982) Responses of plants to low, nonfreezing temperatures: proteins, metabolism, and acclimation. Annu Rev Plant Physiol **33:** 347–372
5. **Guy CL, Hummel RL, Haskell D** (1987) Induction of freezing tolerance in spinach during cold acclimation. Plant Physiol **84:** 868–871
6. **Guy CL, Haskell D** (1987) Induction of freezing tolerance in spinach is associated with the synthesis of cold acclimation induced proteins. Plant Physiol **84:** 872–878
7. **Higgins CM, Manners JM, Scott KJ** (1985) Decrease in three messenger RNA species coding for chloroplast proteins in leaves of barley infected with *Erysiphe graminis* f. sp. hordei. Plant Physiol **78:** 891–894
8. **Kimpel JA, Key J** (1985) Heat shock in plants. Trends Biochem Sci **3:** 353–358
9. **Lawton MA, Lamb CJ** (1987) Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. Mol Cell Biol **7:** 335–341
10. **Logemann J, Schell J, Willmitzer L** (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem **163:** 16–20
11. **Maniatis T, Fritsch EF, Sambrook J** (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. **Mayer JE, Hahne G, Palme K, Schell J** (1987) A simple and general plant tissue extraction procedure for two-dimensional gel electrophoresis. Plant Cell Rep **6:** 77–81
13. **Meza-Basso L, Alberdi M, Raynal M, Ferrero-Cadinanos M-L, Delseny M** (1986) Changes in protein synthesis in rapeseed (*Brassica napus*) seedlings during a low temperature treatment. Plant Physiol **82:** 733–738
14. **Mohapatra SS, Wolfraim L, Poole RJ, Dhindsa RS** (1989) Molecular cloning and relationship to freezing tolerance of cold-acclimation-specific genes of alfalfa. Plant Physiol **89:** 375–380
15. **Moon E, Kao T-H, Wu R** (1987) Rice chloroplast DNA molecules are heterogeneous as revealed by DNA sequences of a cluster of genes. Nucleic Acids Res **15:** 611–630
16. **Mulligan RM, Maloney AP, Walbot V** (1988) RNA processing and multiple transcription initiation sites result in transcript size heterogeneity in maize mitochondria. Mol Gen Genet **211:** 373–380
17. **Mundy J, Chua N-H** (1988) Abscisic acid and water stress induce the expression of a novel rice gene. EMBO J **7:** 2279–2286
18. **Niesbach-Klösgen U, Barzen E, Bernhardt J, Rohde W, Schwarzsommer Z, Reif H J, Wienand U, Saedler H** (1987) Chalcone synthase genes in plants: a tool to study evolutionary relationships. J Mol Evol **26:** 213–225
19. **Oelmüller R** (1989) Photooxidative destruction of chloroplasts and its effects on nuclear gene expression and extraplastidic enzyme levels. Photochem Photobiol **49:** 229–239
20. **Oelmüller R, Mohr H** (1985) Specific action of blue light on phytochrome-mediated enzyme synthesis in the shoot of milo (*Sorghum vulgare* Pers.). Plant Cell Environ **8:** 27–31
21. **Peterson LW, Huffaker RC** (1975) Loss of ribulose bisphosphate carboxylase and increase in proteolytic activity during senescence of detached primary barley leaves. Plant Physiol **55:** 1009–1015
22. **Rivin CJ, Cullis CA, Walbot V** (1986) Evaluating quantitative variation in the genome of *Zea mays*. Genetics **113:** 1009–1013
23. **Springer B, Werr W, Starlinger P, Bennet DC, Zokolica M, Freeling M** (1986) The *shrunken* gene on chromosome 9 of *Zea mays* L. is expressed in various plant tissues and encodes an anaerobic protein. Mol Gen Genet **205:** 461–468
24. **Vierling E, Key JL** (1985) Ribulose 1-5 bisphosphate carboxylase synthesis during heat shock. Plant Physiol **78:** 155–162
25. **Xie Y, Wu R** (1988) Nucleotide sequence of a ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene (*rbc*S) in rice. Nucleic Acids Res **16:** 7749
26. **Youngblom J, Schloss JA, Silflow CD** (1984) The two β-tubulin genes of *Chlamydomonas reinhardtii* code for identical proteins. Mol Cell Biol **4:** 2686–2696